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Lysostaphin Lysis Procedure for Detection of *Staphylococcus aureus* by the Firefly Bioluminescent ATP Method

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The objective of this study was to examine the use of lysostaphin as an ATP-extracting agent for the estimation of *Staphylococcus aureus* cell number by a rapid bioluminescent ATP method. The results of the study showed that (i) lysostaphin (22 U/ml) was able to lyse most of the *S. aureus* cells (>99.9%) at room temperature in 1 min; (ii) ATP of *S. aureus* cells extracted by the lysostaphin lysis procedure was stable for 24 h in the presence of EDTA; (iii) there was a linear relationship between the ATP content and the number of *S. aureus* cells (ranging from 10^4 to 10^6 CFU/ml); and (iv) the lysis of *S. aureus* cells by lysostaphin allowed estimation of the number of *S. aureus* cells in mixed cultures and in meat samples.

Microbiological examination of food products is an important aspect of good manufacturing practice. Unfortunately, traditional methods for microbiological examination of food products take several days. Since time is an important element, many studies have been performed to develop more rapid methods to estimate microbial populations. For example, one method estimates the number of microbial cells based on the determination of ATP levels by bioluminescence (2, 8, 9; K. J. Littel, S. Pikelis, T. Tsai, and A. Spurgash, Abstr. 44th Annu. Meet. Inst. Food Technol., 1984). This method is based upon the following assumptions: (i) every living cell has ATP; (ii) the ATP content of microbial cells is relatively constant across all phases of growth (A. J. D'Eustachio and G. V. Levin, Bacteriol. Proc., p. 121, 1967); and (iii) the light emitted from the bioluminescent reaction is proportional to the ATP concentration (5).

Both determination of the total microbial population and identification of food-borne pathogenic microorganisms are important. A rapid, sensitive, and reliable method as an alternative to the traditional methods for identification of food-borne pathogenic microorganisms would be helpful to the food industry. One of the most common food-borne pathogenic microorganisms is *Staphylococcus aureus*.

Lysostaphin, isolated and described by Schindler and Schuhardt, is capable of lysing *S. aureus* cells rapidly (1, 4, 6, 7). Sensitivity of 38 *S. aureus* isolates to lysostaphin was tested by Heddaeus and his co-workers (3). They reported that 37 strains were susceptible to lysis. Severance and his co-workers studied the lysing power of lysostaphin on 108 strains of *S. aureus* and 60 non-*S. aureus* species (7). They found that 106 of 108 *S. aureus* strains (98%) were susceptible to the lytic action of lysostaphin. However, all of the non-*S. aureus* species were resistant. They also examined the sensitivity to lysostaphin lysis of 100 blood or broth cultures which had been identified as *S. aureus*, other species of staphylococci, micrococci, and streptococci. They reported that all of the cultures identified as *S. aureus* were sensitive to lysostaphin lysis, while the other cultures were resistant. It appears that lysostaphin is specific for *S. aureus*.

In this study, the potential use of a lysostaphin lysis procedure for extraction of the ATP of *S. aureus* cells and use of a rapid bioluminescence firefly method for estimation of the *S. aureus* concentration were evaluated. The study was performed first in pure culture and then in meat samples.

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MATERIALS AND METHODS

Test organisms, media, and buffer. In this study, *Micrococcus luteus*, *Escherichia coli*, *Bacillus subtilis*, and 14 strains of *S. aureus* were used as test organisms. Tryptic soy broth served as the growth medium, while tryptic soy agar and Baird-Parker agar were the standard plate-counting media. Hanks Tris-balanced salt (HTBS) buffer solution (pH 7.75) was used as a buffer during ATP extraction by the lysostaphin lysis procedure. It was prepared by dissolving 8.9 g of Hanks balanced salt without sodium bicarbonate and phenol red (Sigma Chemical Co., St. Louis, Mo.), Trizma-hydrochloride (1.818 g), Trizma base (0.417 g), and disodium salt of EDTA (1.52 g) in 1 liter of distilled water. The pH of the HTBS buffer solution was then adjusted to 7.75 with NaOH, and the solution was sterilized by autoclaving. Peptone-water (0.1%) was used in the dilution blanks for the standard plate-counting procedure.

Reagents. The lysostaphin solution as an ATP-extracting agent was prepared by reconstituting the lyophilized lysostaphin (specific activity, 220 U/mg; Sigma) with HTBS buffer solution. The firefly luciferase-luciferin enzyme solution was prepared by rehydrating Picozyme F (Packard Instrument Co., Downers Grove, Ill.) with 8 ml of degassed sterile distilled water. Standard ATP solution for the calibration curve was prepared by dissolving lyophilized ATP (Picochec; Packard Instrument Co.), 1.1×10^4 ng, with 2 ml of HTBS buffer solution. A different ATP solution was prepared from 990 ng of ATP (Packard) by dissolving in 6 ml of HTBS buffer solution as an internal ATP standard.

Industrial Butterfield (IB) buffer, monobasic potassium phosphate (0.3 mM KH_2PO_4, pH 7.2) solution, was prepared as a dilution buffer for the meat samples. Picoex S buffer (HTBS buffer without EDTA) was prepared as above except that the pH of the buffer was adjusted to 7.0 with HCl and 1.5 g of saponin was added to 1 liter of buffer before filter sterilization. An ATPase reagent, Picoenzyme cocktail (Pico E/C; Packard) and Picozyme F enzyme solutions for background ATP depletion in meat samples, was prepared by dissolving lyophilized Pico E/C with 55 ml of Picoex S buffer; lyophilized Picozyme F was rehydrated with 3 ml of degassed sterile distilled water.

TABLE 1. Lysis of *S. aureus* MF-31[a]

Time (min)	% Lysis at given lysostaphin concn (U/ml)							
	1.1	2.2	4.4	6.6	8.8	11	22	44
1	2.39	57.75	88.13	91.34	86.84	98.20	99.91	99.93
2	5.26	66.51	77.99	97.82	96.09	99.18	99.92	99.99

[a] *S. aureus* cells (10^9) were suspended in HTBS buffer (pH 7.75) and were lysed at room temperature.

Culture preparation. *S. aureus*, *E. coli*, and *B. subtilis* cultures were inoculated from stock cultures on tryptic soy agar slants into 100 ml of tryptic soy broth and incubated at 35°C for 10 to 12 h. *M. luteus* was grown in tryptic soy broth at 25°C for 48 h. The cultures were harvested by centrifugation at 16,000 × *g* for 10 min, and the cells were washed once, suspended, and diluted to the desired cell concentrations with the HTBS buffer solution.

Meat sample preparation. A 10-g portion of ground beef obtained from a local grocery store was stomached with 89 ml of IB buffer and 1 ml of *S. aureus* cell suspension in a sterile stomach bag for 30 s. The stomached samples were filtered through glass-wool filtering fiber and diluted five times with IB buffer.

ATP assay. To extract the bacterial ATP by the lysostaphin lysis procedure with pure cultures, the cell suspensions were mixed with lysostaphin solution in a disposable borosilicate test tube (12 by 75 mm) and incubated at room temperature. Triplicate lysate samples (0.3 ml) were pipetted into assay vials (12 by 55 mm) and placed into a Picolite 6200 Luminometer (Packard). The instrument was programmed to inject first 0.2 ml of Picozyme F and then 0.1 ml of internal ATP standard and to count for 15 s after a 5-s delay. The ATP content of the samples was calculated by a computer (model 85A; Hewlett-Packard, Corvallis, Ore.) connected to the luminometer, based on the data generated from the bioluminescence reaction and coefficients of the calibration curve prepared before running the experiment. The amount of ATP was given as femtograms (10^{-15} g) per unit volume of sample.

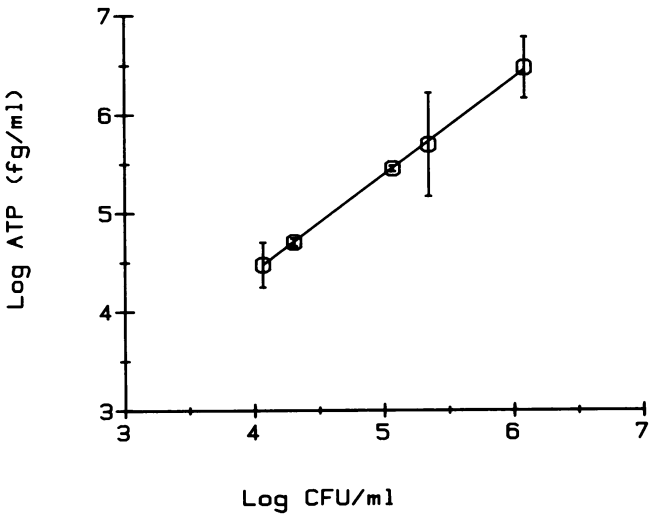

FIG. 1. CFU standard curve. Each point represents the average of the results with 14 strains of *S. aureus*. Error bars show percent deviations. The standard error of estimate and the correlation coefficient of the standard curve are 0.0219 and 0.9997, respectively.

For meat samples, ATP extraction with the lysostaphin lysis procedure was performed on an Acrodisc filter (0.45-μm pore size; Gelman Sciences, Inc., Ann Arbor, Mich.). A 5-ml amount of the meat sample was filtered through a nylon mesh (1-μm pore size) and an Acrodisc filter attached to a 10-ml sterile syringe. After the nylon mesh was discarded, the Acrodisc filter was washed with 5 ml of IB buffer. The Acrodisc filter was incubated with 1 ml of either Pico E/C at 35°C for 30 min or Picozyme F enzyme solution at room temperature for 10 min to deplete background ATP (somatic ATP) present in the meat sample. The filter was washed with 10 ml of IB buffer and 10 ml of HTBS buffer. After the Acrodisc filter was transferred to a 5-ml sterile syringe, the ATP of the bacterial cells was extracted by incubating the filter with 1.2 ml of lysostaphin solution (22

TABLE 2. Stability of ATP in *S. aureus* MF-31 cell lysate and in HTBS-Lysostaphin solution[a]

Time (h)	Sample[b]	Cell lysate				ATP in HTBS-lysostaphin			
		With EDTA		Without EDTA		With EDTA		Without EDTA	
		ATP (fg/ml)	% ATP degradation	ATP (fg/ml)	% ATP degradation	ATP (fg/ml)	% ATP degradation	ATP (fg/ml)	% ATP degradation
0	A	4.76×10^6		4.04×10^6		5.07×10^6		4.97×10^6	
	B	9.71×10^5				5.49×10^5		5.27×10^5	
	C	4.95×10^5		2.35×10^5					
	D	9.99×10^4							
	E	4.67×10^4							
6	A	4.40×10^6	7.56	2.71×10^6	32.92	4.64×10^6	8.48	3.45×10^6	30.58
	B	8.80×10^5	9.37			4.95×10^5	9.84	3.43×10^5	34.91
	C	4.38×10^5	11.52	1.56×10^5	33.62				
	D	8.32×10^4	16.72						
	E	3.73×10^4	20.13						
24	A	4.32×10^6	9.24	1.21×10^6	70.05			2.48×10^6	50.10
	B	8.34×10^5	14.11			4.45×10^5	18.94	2.14×10^5	59.39
	C	4.09×10^5	17.37	8.92×10^5	62.04				
	D	7.22×10^4	27.37						
	E	3.29×10^4	29.55						

[a] *S. aureus* cells were lysed with lysostaphin (22 U/ml) at room temperature. HTBS with and without EDTA, pH 7.75, were used as buffer solutions.
[b] Five dilutions per sample, designated by capital letters, were prepared.

TABLE 3. Determination of cell number of *S. aureus* in mixed culture

Mixed culture	CFU	Ratio of mixed culture	CFU/ml		% of actual
			Actual	Estimated	
S. aureus MF-31 + *E. coli*	10^6	9:1	1.97×10^6	1.53×10^6	78
		5:5	1.64×10^6	8.97×10^5	55
		1:9	3.50×10^5	2.63×10^5	75
	10^5	9:1	2.46×10^5	1.50×10^5	61
		5:5	1.30×10^5	9.33×10^4	72
		1:9			
S. aureus MF-31 + *B. subtilis*	10^5	9:1	2.73×10^6	1.45×10^6	53
		5:5	1.52×10^6	8.81×10^5	58
		1:9	3.77×10^5	1.96×10^5	52
	10^5	9:1	2.80×10^5	1.57×10^5	56
		5:5	1.43×10^5	8.61×10^4	60
		1:9	3.10×10^4	2.20×10^4	71
S. aureus MF-31 + *M. luteus*	10^6	9:1	2.57×10^6	2.92×10^6	114
		5:5	1.29×10^6	1.67×10^6	130
		1:9	3.07×10^5	3.65×10^5	119
	10^5	9:1	3.00×10^5	3.27×10^5	109
		5:5	1.53×10^5	1.64×10^5	107
		1:9	3.37×10^4	3.69×10^4	110

U/ml) at room temperature for 1 min and then pushing the lysostaphin solution slowly through the filter. After each step, the filter was dried by pushing air through the filter twice. Lysate was collected in a small test tube and the ATP assay was performed as described above. As a blank, the IB buffer was filtered instead of the sample.

Actual cell count by standard plate counting. Triplicate tryptic soy agar plates in pure culture and triplicate Baird-Parker agar plates in mixed culture experiments and in meat samples were used to determine the *S. aureus* cell concentration as CFU per unit volume of sample. The plates were counted after incubation at 35°C for 24 to 48 h.

RESULTS AND DISCUSSION

To determine the optimum lysing conditions for ATP extraction, various concentrations of lysostaphin and various incubation times were tested at room temperature with a representative strain, *S. aureus* MF-31. To confirm lysis of *S. aureus* cells and extraction of ATP and other cell materials, the optical density of the cell suspension at 600 nm and that of the supernatant of the cell suspension at 260 nm were determined before and after treatment of the cell suspension with lysostaphin. The decrease in optical density of the cell suspension and the increase in optical density of the lysate supernatant following lysostaphin treatment indicated that treatment with lysostaphin resulted in cell lysis (data not presented). Plate counts were also performed before and after lysostaphin treatment to determine the percent lysis (Table 1). The percent lysis was calculated by dividing the difference between the cell counts obtained prior to and following treatment with lysostaphin by the count obtained prior to treatment, multipled by 100. The lowest lysostaphin concentration which gave >99.9% lysis in the shortest time of incubation (22 U of lysostaphin per ml, 1 min of incubation) was chosen as the lysing condition for ATP extraction (Table 1). A 22-U/ml amount of lysostaphin and 1 min of lysing time were further tested with nine additional strains of *S. aureus*. These lysing conditions also resulted in >99.9% lysis for all the test strains (data not presented).

TABLE 4. *S. aureus* estimation in meat by firefly luminescent method

Enzymes[a]	Cell concn[b] (CFU/g)		% of actual
	Actual	Estimated	
Pico E/C	2.18×10^6	1.61×10^6	74
	4.15×10^5	1.96×10^5	47
Picozyme F	2.18×10^6	1.92×10^6	88
	4.15×10^5	2.05×10^5	49

[a] Enzymes used to deplete background ATP.
[b] Actual and estimated cell concentrations are the averages of triplicate plate counts and duplicate samples, respectively.

After determining the conditions of the lysostaphin lysis procedure, experiments were performed to test the stability of ATP in cell lysates and in HTBS-lysostaphin solution. In these experiments, the effect of EDTA on the stability of ATP was also tested by using HTBS buffer solution with and without EDTA. *S. aureus* MF-31 cell lysate and Picochec ATP solution (see Materials and Methods) were used as sources of ATP in cell lysate and in HTBS-lysostaphin solution, respectively. The amount of ATP per milliliter of sample was determined in the Picolite 6200 Luminometer at 0-, 6-, an 24-h time periods. The percent degradation of ATP at 6 and 24 h was estimated with respect to the initial amount of ATP measured at zero time. The results of the experiments suggested that the presence of the EDTA in the HTBS buffer solution is necessary to reduce ATP degradation (Table 2). The amount of ATP degradation was apparently dependent upon the initial level of ATP (Table 2). These results indicated that the ATP stability was sufficient for the ATP determination in the Picolite 6200 Luminometer.

Following determination of ATP stability, experiments were performed to prepare a standard curve which compared the level of ATP (femtograms per milliliter) with the *S. aureus* cell concentrations (CFU per milliliter) obtained from the plate counts. In these experiments, the ATP level and the cell concentration of 14 strains of *S. aureus* were determined at five different cell levels (10^4 to 10^6 CFU/ml) and the results were plotted as log ATP (femtograms per milliliter) versus log cell concentration (CFU per milliliter) (Fig. 1). The high

correlation level ($r > 0.9$) confirmed that there was a linear relationship between the level of ATP and cell concentration.

Experiments were also performed to estimate the number of *S. aureus* MF-31 cells mixed with *E. coli*, *B. subtilis*, or *M. luteus* at three different ratios (9:1, 5:5, 1:9) at two different cell levels (10^6 and 10^5 CFU/ml) of the mixed cultures (Table 3). The number of *S. aureus* cells was estimated by interpolating the ATP data from the standard curve. The actual cell number of *S. aureus* MF-31 cells in the mixed culture was determined from triplicate Baird-Parker agar plate counting following 48 h of incubation at 35°C. The estimated cell count was then compared with the actual count. The results of the experiments showed that the estimated cell number of *S. aureus* MF-31 for the mixed culture containing *E. coli* was 68.2% of the actual count, while that of the *B. subtilis* mixture was 58.3%. For the mixed culture of *S. aureus* MF-31 and *M. luteus*, the estimated cell number of *S. aureus* was 114.8% of the actual count. On the basis of these results, it was concluded that the lysis of *S. aureus* by lysostaphin was sufficiently specific such that the presence of other microorganisms did not influence the estimated cell count of *S. aureus*.

Although this method works in pure culture, *S. aureus* ATP extraction by the lysostaphin lysis procedure and ATP determination by the firefly luminescent method might be influenced in a food system. For example, meat has somatic ATP that could influence the bacterial ATP measurement. Detection of *S. aureus* cells in meat samples was also tested to determine the potential use of the method in food systems. Pico E/C and Picozyme F enzyme solutions were used to eliminate the background ATP in meat.

The results showed that estimated cell counts were close to the actual cell counts in the meat samples (Table 4). The background ATP in meat and the activity of Pico E/C and Picozyme F to deplete the background ATP were determined by measuring the ATP level of the meat sample in the presence and absence of Pico E/C and Picozyme F without lysostaphin treatment. The result indicated that the meat sample had 1.42×10^5 fg of background ATP per g, and both enzyme systems were able to eliminate the background ATP.

This study shows that estimates of *S. aureus* cells in food is possible with this method. However, the method may need modification when applied to different food systems, depending upon the characteristics of the food.

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